

## NEPHROTOXICITY OF A SINGLE DOSE OF CYCLOPHOSPHAMIDE AND IFOSFAMIDE IN RATS

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**Abstract:** Oxazaphosphorines (cyclophosphamide – CP, ifosfamide – IF) are alkylating cytostatics used in chemotherapy of cancer and autoimmune diseases. They have numerous adverse effects, including uro- and nephrotoxic, dependent of the type of drug, time of therapy and presence of any coexistent nephrotoxic factors in a treated patient. Purpose of this study was to estimate the renal function and the level of urinary bladder dysfunction occurring in rats following administration of a single, large CP/IF dose. The experiment involved 30 rats who were administered a single intraperitoneal dose of 150 mg/kg b.w. of CP (group 1) or IF (group 2) or normal saline (group 3 – control), respectively. Following the administration animals were placed in individual metabolic cages. 24 h later rats were sacrificed, blood collected and nephrectomy and cystectomy performed in order to prepare specimens for histopathological analysis. Circadian diuresis was also assessed, along with a qualitative urine assessment with strip tests and laboratory renal function parameters in plasma and urine: sodium, urea, creatinine and uric acid levels, and circadian elimination of sodium, potassium, urea, creatinine, uric acid and protein. Creatinine clearance, urea clearance and fractionated sodium elimination (FENa) and renal failure index (RFI) were also calculated. An increased diuresis and acidification of urine with reduced circadian elimination of potassium and a significant proteinuria, as well as increased plasma levels of creatinine and urea were found in the group of rats that received a single dose of CP, compared to control animals. Rats treated with IF also demonstrated acidification of urine, reduced circadian potassium elimination, a significant proteinuria and increased plasma creatinine and urea levels, but their diuresis was comparable to that observed in control animals. IF-treated animals were also characterized by reduced urea clearance, FENa and RFI. Histopathological analysis confirmed presence of inflammatory changes in urinary bladders in both groups 1 and 2, and absence of any significant morphological disorders in kidneys. Obtained results suggest a dysfunction of distal tubules and collective tubules developing as a result of administration of a single nephrotoxic dose of IF/CP. FENa and RFI results indicate also a higher nephrotoxic potential of IF administered as a single dose.

**Keywords:** cyclophosphamide, ifosfamide, nephrotoxicity, rats

Alkylating cytostatics belonging to the group of oxazaphosphorines (cyclophosphamide – CP, ifosfamide – IF) are broadly used in chemotherapy of cancer (both solid tumors and proliferative conditions of the hematopoietic system), and in pharmacotherapy of chronic inflammatory diseases and autoimmune conditions (primary systemic vasculitis, visceral lupus, periarteritis nodosa) (1, 2). General adverse effects of those agents include nausea and vomiting, alopecia, hypersensitivity reactions and myelosuppression (3). Long-term administration of CP/IF is associated with a toxic effect on gonads and sterility, and with a paradoxically

increased risk of many types of cancer (prostatic, pulmonary, cystic, breast, endometrial, thyroid, hepatic, gastric) (4). Moreover, chronic use of large CP/IF doses (on the level of 60 mg/kg a day) resulted in development of myocarditis, increased arrhythmogenesis and congestive heart failure, and with interstitial pulmonary fibrosis (4, 5). In case of ifosfamide, a neurotoxic effect was also reported, manifested with signs of encephalopathy, usually disappearing following completion of the treatment (6, 7).

One of the most significant adverse drug reactions (ADR) associated with oxazaphosphorines,

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and particularly with cyclophosphamide, is hemorrhagic cystitis initiated by urotoxic acrolein released in course of CP/IF metabolism. The endogenous, hepatic bio-transformation of cyclophosphamide is based mostly on cytochromes CYP2B6, CYP2C9 and CYP3A4 (8). Metabolism of cyclophosphamide with those cytochromes leads to final production of a 4-hydroxy derivative, that undergoes conversion to aldophosphamide releasing nitrogen mustard – a compound attributed the main cytostatic and alkylating properties of cyclophosphamide (8). Acrolein is also formed in course of those transformations. It is a highly reactive,  $\alpha,\beta$ -unsaturated aldehyde that causes reduction of the intracellular level of glutathione and of other compounds containing thiol groups, and combining with nucleophilic compounds. Acrolein is a strong inducer of lipid peroxidation, and changing the intracellular redox balance leads to dysregulation of cytokines that participate in inflammatory processes, proliferation and apoptosis (9). In the urinary bladder the compound induced a cascade of inflammatory changes, release of numerous proinflammatory mediators and free radicals that damage the urothelium. This disturbance is characteristic particularly to cyclophosphamide, as its chemical structure makes it specifically prone to acrolein generation. A detailed pathophysiological description of oxazaphosphorine-triggered cystitis may be found in review articles (3, 10-13), including the one published by us (14). Similarly, another significant ADR characteristic for CP/IF is nephrotoxicity. Its pathogenesis is also associated with effects dependent on acrolein, synthesized with cytochromes CYP2B6 (15, 16) and CYP2C9 (17), present also in extrahepatic tissues, e.g., in kidneys.

Chloroacetic aldehyde, released along with acrolein as a result of biotransformation of oxazaphosphorines in course of renal injury, is nephrotoxic, especially for proximal and collecting renal tubules. The compound intensifies the unfavorable effect of acrolein causing impairment of function of cells in renal tubules by further reduction of glutathione, acetyl-coenzyme A and ATP levels, and block of the enzymatic complex of NADH ubiquinone oxidoreductase – a component of the mitochondrial respiratory chain (3). Considering the fact that during its bio-transformation ifosfamide generates 40-times more acetic aldehyde than the similar dose of cyclophosphamide, it is believed that nephrotoxicity is a principal, although not sole, adverse effect of that oxazaphosphorine derivative (18).

A clinical manifestation of nephrotoxic effect of oxazaphosphorines covers a broad spectrum of disorders dependent on the type of drug (CP/IF), dose, administration route, time of therapy and some addi-

tional factors (patient age, coexistence of other nephrotoxic factors). Literature data indicate development of both glomerulopathy and tubulopathy, and even of renal failure in CP/IF-treated patients (19, 20).

Considering a variable character of renal dysfunctions and the fact that – as mentioned above – nephrotoxicity is a common ADR observed for both agents (although more characteristic for IF, as cystitis is the dominating adverse effect of CP), the aim of the study was to estimate the renal function and the level of urinary bladder injury in rats receiving a single, large dose of CP/IF.

## EXPERIMENTAL

The medical experiment described in this paper was approved by the 1st local Ethical Commission in Krakow (No. 1/2016). The experiment was consistent with the EU Directive 2010/63 on the protection of animals used for scientific purposes and with the Polish legislation – The Law of 15 January 2015 on the protection of animals used for scientific or educational purposes (Journal of Laws, 26 February 2015, item 266).

### Test groups of animals

Ten-week old (mature) albino Wistar rats of both sexes in equal proportions, with initial body weight of 243.3 g were used in the experiment. Animals were obtained from the Central Animal House of the Pharmaceutical Faculty of the UJCM. After being transported to the local Animal House of the Chair of Pathophysiology UJCM, animals were acclimatized for 7 days. During that time they were kept in collective cages of five animals of the same sex. Duration of acclimatization was consistent with literature data recommending the period of 5-15 days (21). Animals were handled and clinically observed during that period. On the day 8, animals were transferred to a collective pen and randomized into study groups (of 10 animals each, with an equal proportion of both sexes):

Group 1 – rats receiving cyclophosphamide at the single dose of 150 mg/kg b.w.

Group 2 – rats receiving ifosfamide at the single dose of 150 mg/kg b.w.

Group 3 – control – rats receiving normal saline

All animals completed the study. Therefore, 30 animals participated in the experiment.

### Plan of the experiment

The principal part of the experiment was completed using metabolic cages allowing measurement

of circadian diuresis and consumption of feed, in an air-conditioned pen at constant temperature of 22°C and relative humidity of 60%, with a 12/12 h day/night cycle (light phase 8 a.m. – 8 p.m.; dark phase 8 p.m. – 8 a.m.). Following intraperitoneal administration of CP/IF/normal saline, respectively, to animals in groups 1, 2 and 3, study animals were placed in individual metabolic cages for 24 hours, with unlimited but monitored access to water and standard feed (Labofeed, Kcynia, Poland). Before administration of a drug/normal saline animals were weighed using a standard laboratory scales in order to determine the required CP/IF dose and placed in a tamer for rectal measurement of body temperature using a digital thermometer for rodents (Vivari, UK). Selection of the dose of 150 mg/kg b.w. for the applied nephrotoxic CP dose was consistent with literature data, recommending a single administration of 100 mg/kg b.w. (22), 150 mg/kg b.w. (23, 24), or 200 mg/kg b.w. (25, 26) as the amount causing a sub-lethal toxic effect in rats. On the same basis, the recommended single nephrotoxic dose of IF used for experimental purposes ranges between 60–240 in rabbits (27) to as much as 400 mg/kg b.w. in mice (28). Adoption of that dosage regimen results with acute urinary bladder injury developing within 4 – 24 h after CP/IF administration. Both CP and IF were purchased from Sigma Aldrich in crystalline form. Individual doses were prepared by reconstitution in 0.5 mL of normal saline and mixing with a vortex (directly before administration) of an analytically weighed dose calculated according to the body weight of a given animal. Animals in the control group received an intraperitoneal injection with pure normal saline at the same volume of 0.5 mL, as the volume for animals in groups 1 and 2. Keeping animals in metabolic cages allowed the assessment of the following parameters within 24 h after administration of CP/IF/normal saline: body weight [g] (determined by subsequent weighing on an analytical scales), body temperature [°C] (as indicated by subsequent rectal measurement), circadian water consumption [mL/24 h], circadian feed consumption [g/24 h] and circadian diuresis [mL/24 h]. After daily urine output was measured, urine was analyzed using strip tests. Urine was subsequently centrifuged (Heraeus Instruments Megafuge 1.0 R; 2000 rpm – 719 g for 5 min), and divided into portions. Resulting samples were stored at -20°C until laboratory determinations completed with the ADVIA 1200 SIEMENS analyzer. Sodium [mM/L], potassium [mM/L], creatinine (Cr) [mM/L], uric acid [μM/L] and protein [g/L] levels were determined. Having results of circadian diuresis, circadian elimi-

nation of sodium, potassium, urea [mM/24 h], uric acid, creatinine [μM/24 h] and protein [mg/24 h] with urine was calculated. Creatinine clearance was also estimated, according to the formula:

$$CL_{cr} = \frac{Cr \text{ urine } [\mu M/L] \times \text{diuresis } [mL/min]}{Cr \text{ plasma } [\mu M/L]}$$

Urea clearance was calculated according to the formula:

$$CL_{urea} = \frac{\text{urea urine } [mM/L] \times \text{diuresis } [mL/min]}{\text{urea plasma } [mM/L]}$$

Fractional excretion of sodium (FE<sub>Na</sub>) was calculated using the formula:

$$FE_{Na} = \frac{[(Na \text{ urine } [mM/L] \times Cr \text{ plasma } [\mu M/L]) \times 100]}{[(Na \text{ plasma } [mM/L] \times Cr \text{ urine } [\mu M/L])]}$$

The renal failure index (RFI) was calculated with the formula:

$$RFI = \frac{[(Na \text{ urine } [mM/L] \times Cr \text{ plasma } [\mu M/L])] }{Cr \text{ urine } [\mu M/L]}$$

After the period of monitoring in metabolic cages animals were deeply anesthetized by intraperitoneal administration of 60 mg/kg b.w. of sodium pentobarbital (Morbital; Biowet, Puławy). Under general anesthesia blood was collected from the heart in amount allowing completion of all planned laboratory plasma determinations, and study rats were sacrificed by repeated intraperitoneal administration of lethal dose of sodium pentobarbital (200 mg/kg b.w.). Similarly to urine, collected blood was centrifuged and resulting plasma samples were kept frozen for the time of laboratory determinations. Sodium, potassium, urea [mM/L] and creatinine (Cr) and uric acid [μM/L] levels were determined in plasma with the same analyzer as the one used for urine samples. Protein was not assayed in plasma. After definitive cease of vital signs, cystectomy and nephrectomy was performed in study animals in order to obtain tissue necessary for subsequent histopathological assessment of kidneys and urinary bladders.

#### Urine analysis with strip tests

Strip tests (Insight, ACON Laboratories), allowing pH and specific gravity (SG) assessment, glucose level, presence of blood, leukocytes, nitrites, urobilinogen, ketones, bilirubin and protein, were used for qualitative and semi-quantitative assessment of urine obtained from study animals. To perform a test, strips were placed in previously mixed urine samples, excess was filtered out and strips left for 60 s (according to the manufacturer's instructions after that time the result of blood and protein presence could be considered reliable). After that time strips were compared to the scale printed on the package and results were recorded.

**Histological assessment of collected urinary bladders and kidneys**

The histological analysis was performed at the Department of Anatomico-pathology of the Non-public Healthcare Institution „Prosmied” in Kraków. Kidneys and bladders collected in course of the post-mortem examination were washed in normal saline and fixed for 24 hours in 8% formal in phosphate buffer solution (PBS pH 7.4). Collected specimens were subsequently washed under running water for 2 h, and dehydrated in ethanol (condensations increasing from 50 to 100%). Before embedding in paraffin, specimens were passed through xylene solution in order to be cleared. From xylene, specimens were transferred to a 1 : 1 xylene and paraffin mixture and incubated at 37°C for 2 h. Then, individual tissue fragments were transferred twice into pure paraffin and incubated at 62°C. After 2 h, specimens were embedded into paraffin blocks. After setting down blocks were sliced with a microtome, and resulting sections were dried on a glass slide at 37°C. Specimens were stained with hematoxylin-eosin (HE) in order to facilitate the histological assessment of inflammation. Specimens of urinary bladders and kidneys from animals receiving normal

saline (control group 3) were compared to those from animals treated with CP/IF.

The histological analysis was performed using an optical (light) microscope (Delta Optical) at a magnification of 40 × (urinary bladders) and 100 × (kidneys). The microscopic images were taken using a DLT-Cam Basic 2MP microscopic camera and DLTCamViewer software.

**Statistical analysis**

Results of qualitative and semi-quantitative urine analysis completed with strip tests were not subject to statistical assessment. Obtained results of vital signs and laboratory determinations of urine and plasma were initially assessed using the Shapiro-Wilk test, in order to verify normality of distribution. With verified normality of distribution, the essential statistical analysis of differences between groups (group 1 vs. group 3, and group 2 vs. group 3) was performed using the t-Student test, with the accepted statistical significance level of  $p \leq 0.05$ . In those cases when results verified by the initial Shapiro-Wilk test failed to meet criteria of a normal distribution, differences were tested with the Mann-Whitney test, also with the statistical significance level of  $p \leq 0.05$ .

Table 1. Metabolic cage measurement - results (mean ± SD) of the living parameters of the animals used in the experiment.

	Group 1 CP-treated rats	Group 2 IF-treated rats	Group 3 Control rats	Statistical analysis (p value)	
				1-3	2-3
Body weight[g]	248.50 ± 32.30	257.00 ± 10.89	226.6 ± 44.89	NS	NS
Body temperature[°C]	36.23 ± 0.90	35.50 ± 0.25	37.43 ± 0.93	0.04	0.01
Daily water intake[mL/24h]	22.50 ± 11.47	13.50 ± 7.19	26.34 ± 4.07	NS	0.02
Daily feed intake[g/24h]	1.98 ± 1.37	4.53 ± 3.72	23.75 ± 6.16	< 0.001	< 0.001

NS - Non-significant

Table 2. The results of the estimated plasma parameters (mean ± SD) in study rats.

	Group 1 CP-treated rats	Group 2 IF-treated rats	Group 3 Control rats	Statistical analysis (p value)	
				1-3	2-3
Na [mM/L]	141.95 ± 1.26	144.75 ± 1.95	142.06 ± 1.79	NS	0.04
Urea [mM/L]	8.97 ± 1.54	10.93 ± 2.12	6.34 ± 0.75	0.03	< 0.001
Creatinine [µM/L]	38.45 ± 7.96	34.67 ± 7.48	28.85 ± 1.77	0.05	0.05
Uric acid [µM/L]	165.82 ± 45.77	178.38 ± 21.88	157.96 ± 35.14	NS	NS

NS - Non-significant

Table 3. The results of the estimated urine parameters (mean  $\pm$  SD) in study rats.

	Group 1 CP-treated rats	Group 2 IF-treated rats	Group 3 Control rats	Statistical analysis (p value)	
				1-3	2-3
Diuresis[mL/24 h]	16.70 $\pm$ 8.31	8.95 $\pm$ 7.49	5.85 $\pm$ 2.37	0.04	NS
Diuresis[mL/min]	0.012 $\pm$ 0.006	0.006 $\pm$ 0.005	0.004 $\pm$ 0.002	0.04	NS
pH	6.75 $\pm$ 0.29	6.13 $\pm$ 0.75	8.94 $\pm$ 0.18	< 0.001	0.002
SG [g/cm <sup>3</sup> ]	1.014 $\pm$ 0.003	1.023 $\pm$ 0.006	1.011 $\pm$ 0.002	NS	0.007
Na [mM/L]	46.00 $\pm$ 10.94	80.83 $\pm$ 36.08	127.58 $\pm$ 34.67	0.004	NS
K [mM/L]	68.10 $\pm$ 27.31	159.32 $\pm$ 81.20	352.96 $\pm$ 64.66	0.004	0.004
Urea [mM/L]	414.35 $\pm$ 115.32	949.25 $\pm$ 532.44	1003.96 $\pm$ 188.91	0.004	NS
Creatinine [mM/L]	3.35 $\pm$ 1.17	6.75 $\pm$ 4.34	6.10 $\pm$ 1.10	0.008	NS
Uric acid [ $\mu$ M/L]	826.18 $\pm$ 321.76	1378.10 $\pm$ 401.61	1286.54 $\pm$ 296.60	0.05	NS
Urine protein [g/L]	2.5 $\pm$ 0.92	3.91 $\pm$ 1.15	0.81 $\pm$ 0.51	0.008	0.03
Na [mM/24 h]	0.78 $\pm$ 0.44	0.55 $\pm$ 0.14	0.75 $\pm$ 0.37	NS	NS
K [mM/24 h]	1.09 $\pm$ 0.49	1.03 $\pm$ 0.20	2.05 $\pm$ 0.86	0.05	0.02
Urea [mM/24 h]	6.36 $\pm$ 1.91	5.72 $\pm$ 0.61	5.80 $\pm$ 2.38	NS	NS
Creatinine [ $\mu$ M/24 h]	50.49 $\pm$ 18.12	42.21 $\pm$ 13.76	35.19 $\pm$ 13.85	NS	NS
Uric acid [ $\mu$ M/24 h]	13.53 $\pm$ 6.82	10.58 $\pm$ 5.36	7.49 $\pm$ 3.75	NS	NS
Urine protein [mg/24 h]	38.70 $\pm$ 17.12	23.57 $\pm$ 14.83	5.27 $\pm$ 4.47	0.004	0.03

NS - Non-significant, SG - specific gravity

## RESULTS

### Vital signs

After the end of monitoring in metabolic cages, body weight of study animals was not significantly different. 24 h after administration of a drug, animals receiving CP or IF had a significantly lower body temperature compared to control animals. Similarly, a statistically significant reduction of circadian feed consumption was found in both groups 1 and 2 compared to the control, which was probably a result of unfavorable systemic effect of CP/IF, particularly on gastrointestinal mucosa. Moreover, rats receiving IF consumed a significantly lower amount of water within 24 hours post the drug administration, compared to the control.

Detailed results of measurement of vital signs are presented in Table 1. Considering the fact that measurements were characterized by a normal distribution, the statistical analysis of intra-group differences was performed using the t-Student test.

### Qualitative and semi-quantitative urinalysis with strip tests

Values of pH and specific gravity are described below, along with description of parameters measured in urine. A minor leukocyte count (estimated at

15 ( $\pm$ ); n = 7) and a minor proteinuria estimated at (+) (30 [mg/dL]; n = 5) were demonstrated in control animals. Absence of other measured components was confirmed in their urine.

Urine from animals receiving CP was characterized by leukocytosis in 25% of cases estimated at + (70 [leu/ $\mu$ L]), in 25% at ++ (125 [leu/ $\mu$ L]), and in the remaining half of cases at +++ (500 [leu/ $\mu$ L]). Presence of blood was confirmed in all urine samples, estimated at +++. Similarly, proteinuria was found in all animals in the group 1, estimated at +++ (300 [mg/dL]). Nitrites, urobilinogen, ketones, bilirubin or glucose were found in urine from none of CP-treated animals.

Strip urinalysis of rats treated with IF gave similar results: no urobilinogen, ketones, bilirubin or glucose were found, but presence of nitrites was demonstrated in 25% of cases. Leukocytosis was lower than that measured in the group 1: in 25% of cases it was estimated at +/- (15 [leu/ $\mu$ L]), in 25% at + (70 [leu/ $\mu$ L]), and no leukocytes were found in urine in the remaining half of cases. Presence of blood estimated at +++ was demonstrated in half of cases; but no hematuria was found in the other half. Similarly to CP-treated animals, rats treated with IF demonstrated proteinuria, estimated at +++ (300 [mg/dL]) in all cases.

Parameters determined in plasma

Considering signs of hemolysis evident in majority of plasma samples, results of plasma potassium levels had been discarded as non-reliable.

Values of plasma parameters assessed in CP-treated were not significantly different from control animals, except for a statistically significant increased urea and creatinine levels. But IF-treated animals were characterized with a significant hypernatremia, increased plasma urea and creatinine levels, with a statistically insignificant reduction of the plasma uric acid level. Detailed results of measurement of plasma parameters are presented in Table 2. Considering the fact that measurements had no normal distribution, the statistical analysis of intra-group differences was performed using the Mann-Whitney test.

Parameters determined in urine

The circadian diuresis and the calculated minute diuresis was significantly higher in CP-treated animals. On the contrary, IF-treated rats demonstrated no significant difference in diuresis compared to control animals. In both groups of treated animals a significant acidosis of urine was found, compared to the control group. Specific gravity of urine from rats receiving IF was significantly higher compared to the control.

Urine levels of all determined substances were significantly lower in the group 1 (CP treatment)

compared to the control, except for protein. Protein levels were statistically significantly higher in CP-treated animals. IF-treated rats were characterized by significantly lower and higher of potassium and protein levels, respectively, compared to the control.

The analysis of circadian urine elimination of tested substances demonstrated in both groups of treated animals a statistically significant reduction of potassium elimination and a significant proteinuria. Other urine components were eliminated in amounts similar to those observed in the control group.

Detailed results of measurement of plasma parameters are presented in Table 3. Considering the fact that measurements were characterized by a normal distribution, the statistical analysis of intra-group differences was performed using the t-Student test.

Values of calculated parameters: creatinine clearance, urea clearance, fractionated sodium elimination and renal failure index were not significantly different in CP-treated rats compared to the control. On the contrary, comparing IF-treated animals with the control, a statistically significant reduction of urea clearance, FENa and RFI were found. Creatinine clearance was an exception.

The above mentioned relationships are illustrated in Figures 1-4, below (graphs present median values, quartiles and ranges for individual variables).

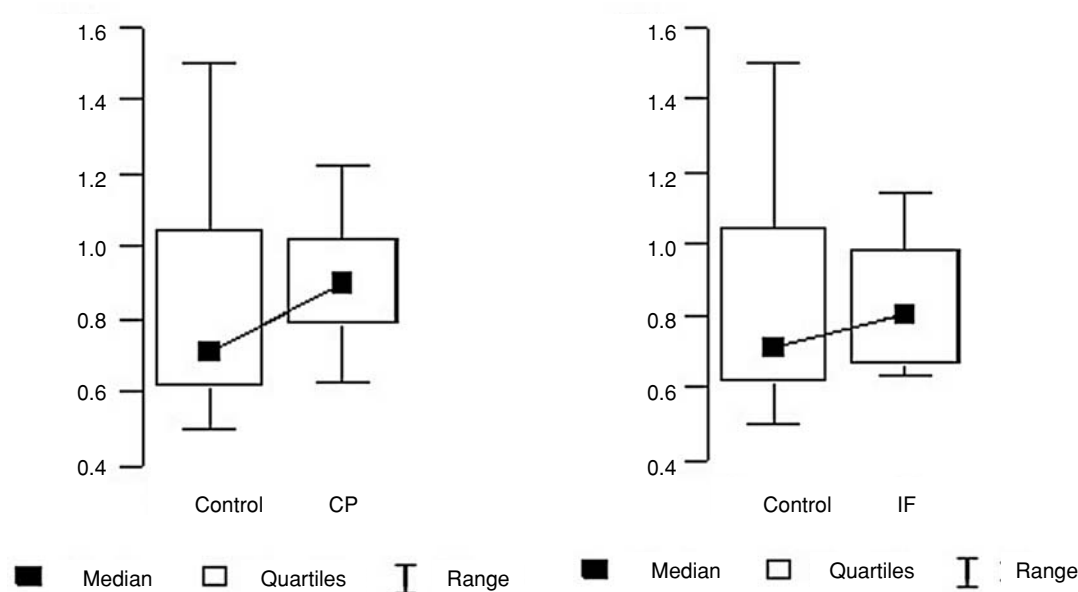


Figure 1. Creatinine clearance in cyclophosphamide-treated (left) and ifosfamide-treated (right) rats (no statistically significant differences revealed in both comparisons)



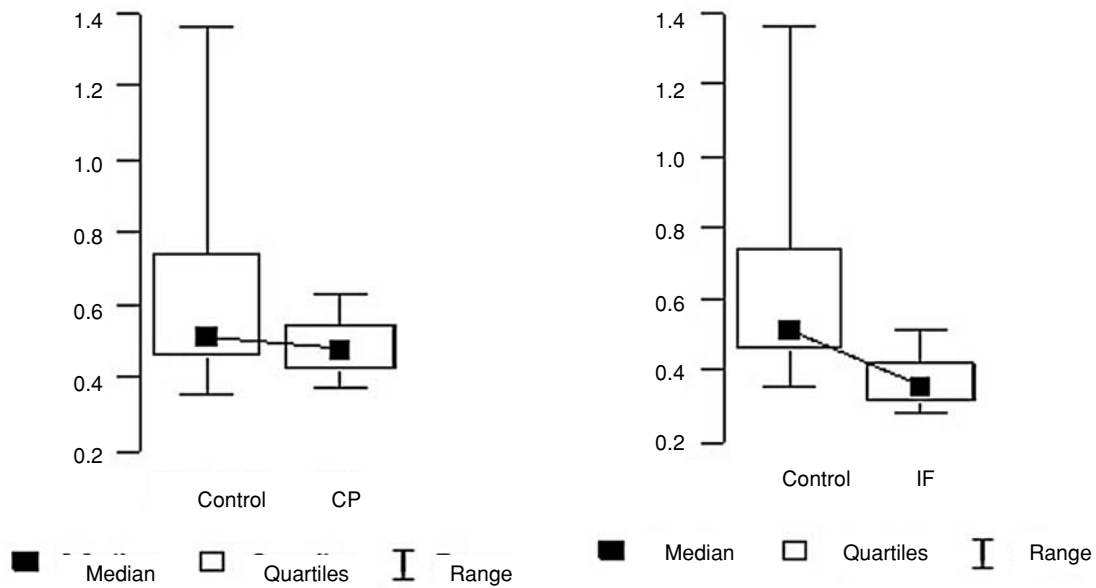


Figure 2. Urea clearance in cyclophosphamide-treated (left) and ifosfamide-treated (right) rats ( $p < 0.05$  for Control-IF rats, non-significant for Control-CP ones)

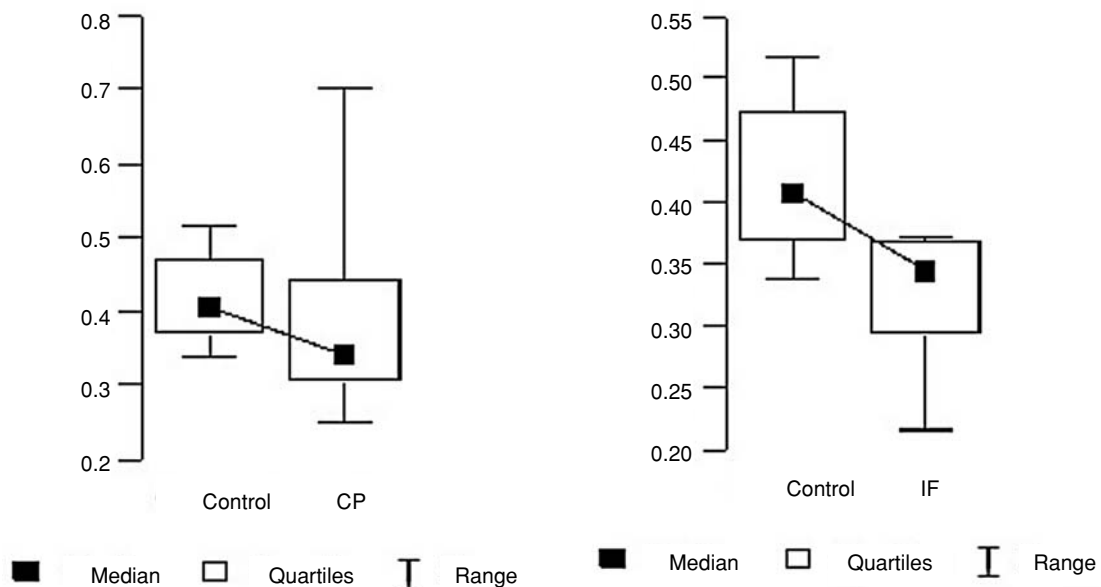


Figure 3. Fractional excretion of sodium (FENa) in cyclophosphamide-treated (left) and ifosfamide-treated (right) rats ( $p < 0.05$  for Control-IF rats, non-significant for Control-CP ones)

### Results of histopathological analysis of urinary bladders and kidneys

Histopathological assessment of urinary bladder specimens from rats receiving a single large dose of CP demonstrated congestion with inflammatory infiltration in stroma of the cystic mucosa, and epithelium with signs of regeneration. Moreover,

clusters of dilated tubules covered by a regular cubic epithelium and a low cylindrical epithelium were visible in selected specimens. Urinary bladders of animals treated with a single large dose of IF presented similar disturbances to those observed in CP-treated rats, with additional signs of exfoliation of the lining epithelium.

Histopathological analysis of kidneys demonstrated minor congestion in both CP and IF-treated animals, but besides that the presentation was within the normal range determined for control animals. The samples of microscopic images of the urinary bladders and kidneys obtained from control and CP/IF treated rats are shown in Figures 5 and 6, respectively.

DISCUSSION

The experiment demonstrated existence of the following principal disorders:

- 1. Rats treated with a single dose of CP demonstrated reduced body temperature and reduced circadian feed consumption, with increased diuresis and decrease of urine pH. That group presented a reduced circadian elimination of potassium and a significant proteinuria, as well as increased plasma creatinine and urea levels, compared to control animals.
- 2. Similarly to CP-treated rats, animals receiving a single dose of IF were also characterized by reduced body temperature and reduced circadian feed consumption, and by urine pH decrease. Contrary to CP-treated animals, that group presented diuresis comparable to that observed in control animals, with a lower daily water consumption. Administration of a single dose of IF resulted in

reduced 24-hour elimination of potassium and a significant proteinuria, just like in case of a single dose of CP. Similarly to animals treated with CP, administration of IF resulted in increased creatinine and urea plasma levels. Moreover, IF-treated animals presented reduced urea clearance values, fractionated sodium elimination and acute renal failure index.

Although being statistically significant, reduced urine levels of all parameters determined in CP-treated animals compared to the control group, were rather a result of increased diuresis in that group of rats, and not a result of a real increase of urine elimination of those substances. Particularly, the analysis of circadian elimination of electrolytes and nitrogen compounds demonstrated a reduced urine elimination of potassium only, with a significant proteinuria in CP-treated animals (and in IF-treated, as well). Considering those laboratory results globally, it should be stated that renal dysfunctions observed after administration of both CP and IF were of functional character and were not accompanied by any morphological injury of kidneys evident in the histopathological assessment. Therefore, a single exposure to oxazaphosphorines was sufficient to cause a functional disorder of kidneys, manifested principally by impaired tubular transport in terms of secretion and reuptake of potassium, and – in case of CP-treated animals – by impaired condensation of urine. Decreased urine potassium elimination may be

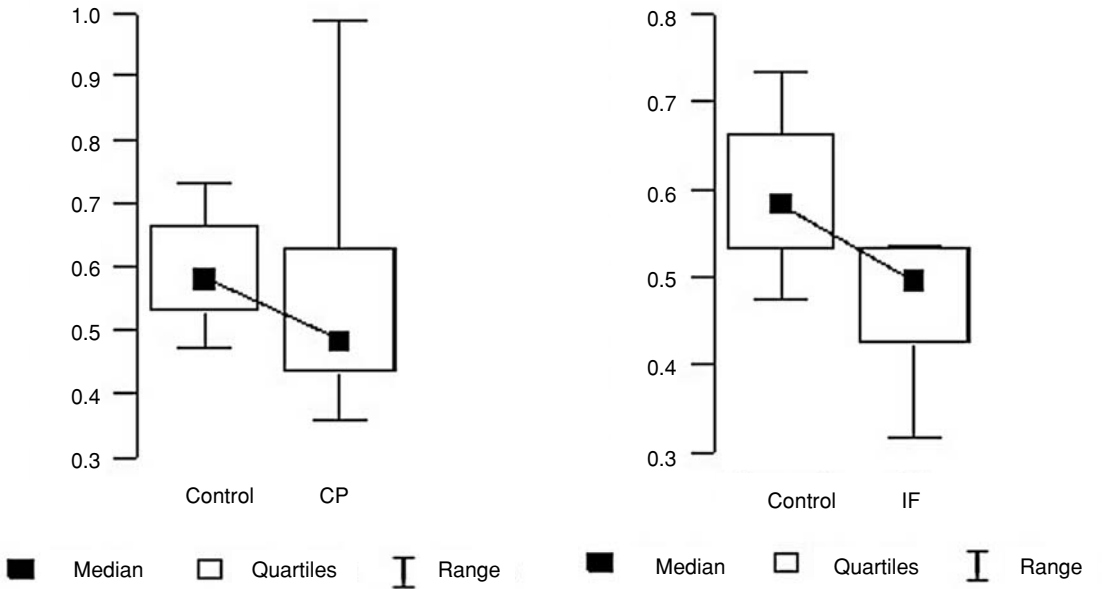


Figure 4. Renal failure index (RFI) in cyclophosphamide-treated (left) and ifosfamide-treated (right) rats ( $p < 0.05$  for Control-IF rats, non-significant for Control-CP ones)



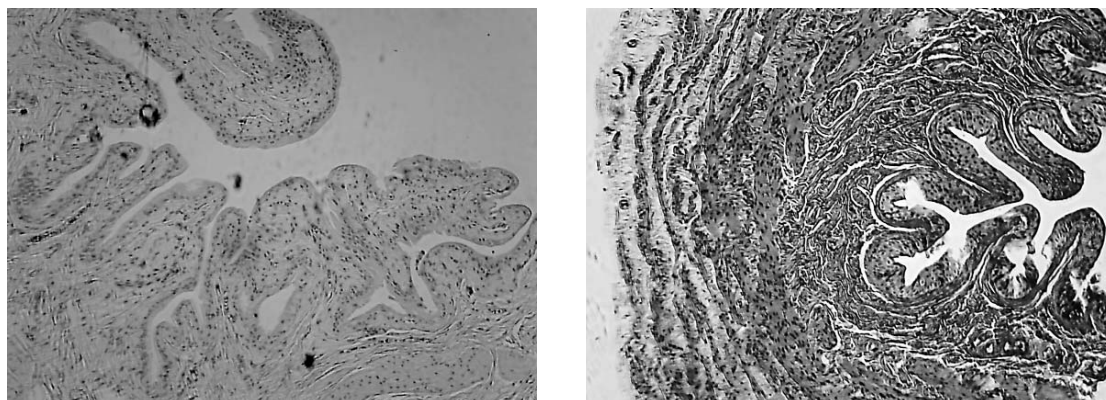


Figure 5. Light microscopy of the urinary bladders – in control (left) and in the CP/IF (right) rats

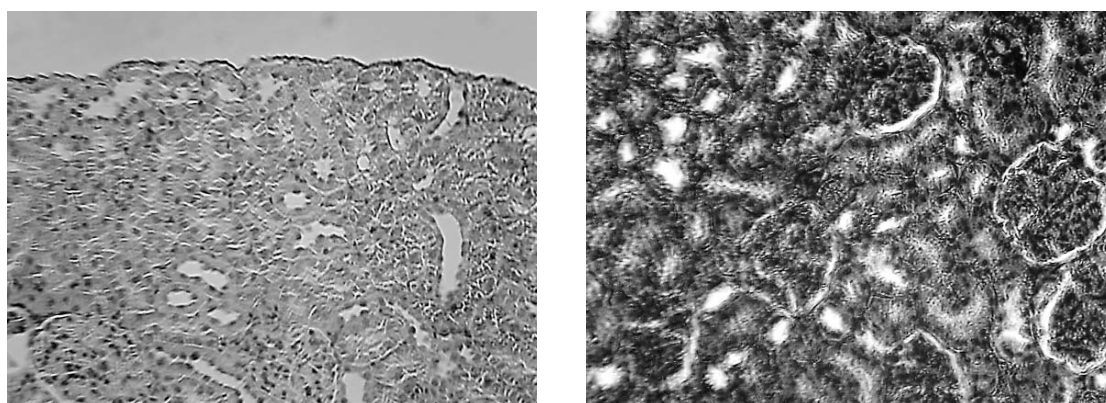


Figure 6. Light microscopy of the kidneys – in control (left) and in the CP/IF (right) rats

associated with an expected retention of the element in blood. That assumption could not be verified because of hemolysis of plasma samples. Regulation of plasma potassium levels (and consequently the dynamics of its elimination with urine) is based mostly on exchange of the ion between intra- and extracellular fluid. Kidneys are a critical organ engaged in regulation of systemic amount of potassium. Within a nephron, potassium ions undergo glomerular filtration, and are subsequently reuptaken in proximal tubules and in the loop of Henly. The final urinary elimination of majority of potassium ions depends on the active secretion of the ion by cells of distal and collecting tubules, determined by increased plasma levels of the electrolyte, increased  $\text{Na}^+ - \text{K}^+/\text{H}^+$  exchange with participation of the aldosterone-regulated ATPase in basal-lateral membrane and a direct secretion of the ion into urine (29, 30). Considering a reduced circadian potassium elimination observed in study animals, a thesis may be formulated that both CP and IF contribute to dysfunction of distal and collecting tubules.

Urine acidification is an additional argument supporting tubulopathy caused by CP/IF. The process is also associated with potassium metabolism, as mentioned above ( $\text{K}^+/\text{H}^+$  exchange). In general terms it may be stated that two processes occur in nephrons: reuptake of alkalis and secretion of acids. The first one takes place in proximal tubules, and the other one in distal tubules (31). Secretion of  $\text{H}^+$  ions into urine takes place *via* the mentioned above antiport  $\text{K}^+/\text{H}^+ - \text{ATPase}$  and the luminal pump  $\text{H}^+ - \text{ATPase}$ , and a final acidification of urine depends also on phosphate and ammonia buffer (32, 33). It seems that CP and IF may impair those mechanisms, leading to final reduction of urine pH and increased potassium reuptake.

Moreover, CP-treated animals demonstrated polyuria, which – in face of circadian water consumption comparable to that observed in the control group – may constitute another argument in favor of dysfunction of distal and collecting tubules. Reuptake of water and final condensation of urine depends on aquaporins – integral proteins of water

channels induced in apical membranes of collecting tubules by antidiuretic hormone (34). Therefore, polyuria observed in the group of rats receiving a nephrotoxic dose of CP may be a result of impairment of that mechanism. That observation is contrary to literature reports indicating a possible development of „syndrome of nephrogenic syndrome of inappropriate antidiuresis” induced by CP as a result of increased expression and hyperactivity of aquaporins in collecting tubules of rats following administration of the dose of 12-96 mg/kg b.w. (35) or 25-50 mg/kg b.w. of CP (36), in 12-72 h after administration of cyclophosphamide. The quoted studies indicate also that the effect may be ADH-independent, and time- and dose-dependent, therefore the effect of CP on the volume of diuresis has to be more precisely defined.

A significant proteinuria was demonstrated in both treated groups. Pathogenesis of that disorder seems complex and may be a result of above mentioned dysfunction of renal tubules, or be a consequence of other disorders. According to the general pathophysiological classification, proteinuria may be of prerenal character (overload), renal character (glomerular or tubular) or extrarenal character (e.g., inflammatory, neoplastic or obstructive disturbances of the lower urinary tract) (37, 38). Therefore, presence of increased urinary elimination of protein in study rats may be a result of impairment of resorptive function of tubules, but considering presence of oxazaphosphorine-induced inflammatory changes in the urinary bladder, it may be also of extrarenal character.

Reduced values of both  $FE_{Na}$  and RFI were also demonstrated in IF-treated rats. Fractional excretion of sodium is defined as the amount of sodium finally eliminated with final urine after the phase of glomerular filtration (39, 40). The renal failure index describes a relationship between the plasma creatinine level and urine sodium and creatinine levels (41). Both  $FE_{Na}$  and RFI are currently regarded modern laboratory parameters of acute kidney injury (AKI) – the term used currently in place of formerly used “acute renal failure”. The disorders is a result of pre-, intra- and extrarenal processes leading to development of acute tubular necrosis and finally to irreversible failure of the organ (42, 43). Clinical guidelines recommend the diagnosis of AKI, along with a possible pathophysiological differentiation into ante- and intrarenal form, based on values of the above mentioned values of  $FE_{Na}$  and RFI, that are decreased in the prerenal form. Although clinical observations cannot be uncritically translated into experimental conditions, reduced values of both

$FE_{Na}$  and RFI were observed in rats receiving a single nephrotoxic dose of IF, compared to corresponding values in control animals (Figs. 3 and 4). Reduced  $FE_{Na}$  in IF-treated animals was accompanied by reduced circadian urinary sodium elimination [mM/24 h]. Although no statistical significance was reached in comparison to control animals, a trend could be observed. No  $FE_{Na}$  or RFI reduction was demonstrated in animals receiving a single, nephrotoxic dose of CP. Therefore, those results may suggest development of functional AKI caused by IF, with normal diuresis comparable to that observed in the control, and a normal creatinine clearance, which does not exclude the presence of AKI – the disorder may occur in oliguric form or with a paradoxical normal diuresis (44). Confirmation of the pathophysiological prerenal character of AKI in those animals (as evidenced by reduced  $FE_{Na}$  and RFI compared to the control), and particularly of the potential mechanism responsible for prerenal functional kidney injury requires some more detailed studies.

## CONCLUSIONS

Multiple renal dysfunctions resulted from oxazaphosphorines administration were reported – a combination of glomerular, proximal or distal tubular damage, Fanconi syndrome and reversible AKI or a permanent chronic kidney disease were revealed. These disturbances are characterized by a considerable variability in the onset, clinical manifestation, severity and reversibility and dependent on the dose and time of the therapy (18-20).

Renal dysfunctions observed in study animals are consistent with the above mentioned literature data. Described disorders developing as a result of administration of a single, nephrotoxic dose of CP/IF also suggest a dysfunction of mostly distal and collecting tubules, with absence of morphological changes in kidneys. Proteinuria, urine acidification with reduced circadian elimination of potassium and increased plasma levels of creatinine and urea were demonstrated as a result of a single exposure to nephrotoxic doses of CP/IF. The commonly recognized lower nephrotoxicity of cyclophosphamide was also confirmed in this study. No laboratory features of AKI (RFI,  $FE_{Na}$ ) were demonstrated following administration of a single dose, contrary to IF-treated rats. However, a significant polyuria was observed in case of CP, in place of the literature-reported antidiuresis syndrome, which may be a result of different methodology of the study, different dosage and observation time.

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